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Posaconazole Prophylaxis in Experimental Azole-Resistant Invasive Pulmonary Aspergillosis

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We investigated the efficacy of posaconazole prophylaxis in preventing invasive aspergillosis due to azole-resistant *Aspergillus fumigatus* isolates. Using a neutropenic murine model of pulmonary infection, posaconazole prophylaxis was evaluated using three isogenic clinical isolates, with posaconazole MICs of 0.063 mg/liter (wild type), 0.5 mg/liter (F219I mutation), and 16 mg/liter. A fourth isolate harboring TR_{34}/L98H (MIC of 0.5 mg/liter) was also tested. Posaconazole prophylaxis was effective in *A. fumigatus* with posaconazole MICs of ≤0.5 mg/liter, where 100% survival was reached. However, breakthrough infection was observed in mice infected with the isolate for which the posaconazole MIC was >16 mg/liter.

Invasive aspergillosis (IA) is an important opportunistic fungal infection, especially among immunocompromised patients, with an overall mortality ranging between 30 and 88% and up to 95% in patients underlying azole-resistant *Aspergillus fumigatus* infections (1–5). Posaconazole (POS) is an extended-spectrum triazole recommended for salvage therapy and prophylaxis of aspergillus diseases in neutropenic patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS) and in patients with graft-versus-host disease (GvHD) (6–8). The incidence of invasive fungal diseases is significantly reduced in patients receiving POS prophylaxis (9, 10).

Over the past decade, acquired azole resistance in *Aspergillus fumigatus* is increasingly recognized as an emerging problem (11, 12). Animal models and clinical experience indicate that infection with an azole-resistant isolate is associated with azole treatment failure (4, 12–27). Although azole resistance may develop during azole therapy, the main route of resistance selection appears through environmental exposure of *A. fumigatus* to azole fungicides (28). Surveillance studies indicate that in areas of endemicity up to two-thirds of patients with azole-resistant aspergillus diseases have no history of azole therapy (4). Therefore, in areas of environmental resistance, any patient at risk for IA can develop azole-resistant disease (29).

POS has been shown to be effective for preventing IA and is becoming a more common strategy to manage invasive fungal infection in high-risk patients (9, 10, 30–32). However, the efficacy of the drug in preventing IA due to azole-resistant isolates is unknown, and to date only one case of breakthrough IA due to azole-resistant *A. fumigatus* has been reported (18). In this study, the patient was diagnosed with AML and developed IA due to an *A. fumigatus* isolate with a TR_{34}/L98H resistance mechanism and a POS MIC of 0.5 mg/liter. The breakthrough infection developed despite adequate POS plasma levels (2.01 mg/liter) (18).

We investigated the impact of azole resistance on the efficacy of POS prophylaxis in preventing IA using an immunosuppressed mouse model of pulmonary aspergillosis.

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MATERIALS AND METHODS

Fungal isolates. Four clinical *A. fumigatus* isolates were studied in a neutropenic murine model of pulmonary infection. Three isolates were obtained sequentially from respiratory samples of a patient with an aspergilloma (33). The first isolate exhibited a wild-type phenotype (POS MIC of 0.063 mg/liter) and harbored an A9T mutation in the CYP51A gene, which was not associated with azole resistance. The patient was treated with itraconazole, during which an F219I mutation was gained that corresponded with itraconazole resistance (MIC of >16 mg/liter) and low POS resistance (MIC of 0.5 mg/liter). The patient was then treated with POS, and a phenotype emerged which was POS highly resistant (MIC of >16 mg/liter) although no additional changes in the Cyp51A gene were found (10). Microsatellite typing indicated that these isolates were isogenic (10). A fourth genetically unrelated isolate harboring the highly prevalent TR_{34}/L98H resistance mechanism with low POS resistance (MIC of 0.5 mg/liter) was also investigated. This isolate was obtained from a patient with proven IA and was previously evaluated in a nonneutropenic murine model of IA (24) (Table 1). Strain identification was confirmed by sequence-based analysis, as described previously (12). The isolates were stored in 10% glycerol broth at −80°C, and the inoculum was prepared as described before (34). An *in vitro* antifungal susceptibility test was performed based on the EUCAST guidelines using a broth microdilution format (35).

***In vitro* kinetic growth assay.** In order to rule out important fitness costs associated with the acquisition of resistance mechanisms, the growth characteristics of the four isolates were determined using a previously described microbroth kinetic system (36). Briefly, the inocula were prepared by diluting an overnight culture grown on agar plates with 0.9% NaCl to 1×10^6 to 5×10^6 CFU/ml. The fungal suspensions were then further diluted in RPMI 1640 medium (with L-glutamine, without sodium bicarbonate) (Sigma, USA) supplemented with 0.165 M morpholinepropanesulfonic acid (MOPS) to give a final inoculum between 0.5 ×

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TABLE 1 Characteristics of four *A. fumigatus* isolates used in the prophylaxis model

Isolate no.[a]	Strain no.	Prior azole exposure	Cyp51A substitution(s)[b]	MIC (mg/liter)[c]			
				AMB	ITC	VRC	POS
1	V74-61	ITC	A9T	1	0.5	1	0.063
2	V76-03	ITC	A9T, F219I	1	>16	1	0.5
3	V79-63	POS	A9T, F219I	1	>16	8	>16
4	V52-35	No	TR_{34}/L98H	1	>16	4	0.5

[a] Isolates 1, 2, and 3 were isogenic and recovered from an aspergilloma patient (29). Isolate number 4 was unrelated to isolates 1 to 3 and was obtained from a patient with proven invasive aspergillosis.

[b] Isolates 1 to 3 harbored an A9T mutation Cyp51A, but this was found not to be associated with a change of the susceptibility to azoles. The F219I mutation was found to be associated with itraconazole resistance (29).

[c] During POS therapy the phenotype changed for voriconazole (VRC) and posaconazole (POS), but this change was not associated with a subsequent mutation in the *cyp51A* gene, suggesting another, yet unknown, resistance mechanism. AMB, amphotericin B; ITC, itraconazole.

10^3 and 2.5×10^3 CFU/ml. Fungal inoculum (100 μl) was added to each well of a sterile 96-well flat-bottomed microtiter plate containing RPMI 1640 medium (100 μl/well). Inoculations were performed in duplicate for each isolate, and two wells contained only medium (background control). After inoculation, the microtiter plates were agitated for 15 s and incubated at 35 to 37°C inside a plate reader (Rosys Anthos HT3; Anthos Labtec Instruments GmbH, Salzburg, Austria) for 72 h under ambient conditions. The optical density at 405 nm (OD_{405}) was automatically recorded for each well every 30 min without shaking. The changes in ODs over time were used to generate growth curves. The background ODs (the ODs of growth control wells) were subtracted from the ODs of the inoculated wells, and then the growth curve was plotted against time.

Infection model. A total of 264 outbred CD-1 (Charles River, the Netherlands) female mice, 4 to 5 weeks old, weighing 20 to 25 g, were randomized into groups of 11 mice for POS prophylaxis. To render the mice neutropenic, cyclophosphamide (150 mg/kg on days −4 and +4 and 100 mg/kg on day −1) was administered. Animals were treated from day −2 to 7 days postchallenge with 1, 4, 8, 16, and 32 mg/kg oral POS (Merck B.V., the Netherlands) once daily. Clinical trials support once- or twice-daily dosing based on the long elimination phase half-life of POS (37).

On day zero and 2 h after POS therapy, mice were infected with the *A. fumigatus* isolates through instillation of the inoculum, corresponding to a 90% lethal dose (LD_{90}) of each isolate, in the nares using a procedure described before (38, 39). The inoculum used was 7×10^6 (V74-61, wild type; POS MIC of 0.063 mg/liter), 3×10^7 (V76-03, F219I mutation; POS MIC of 0.5 mg/liter), 1×10^7 (V79-63, F219I mutation; POS MIC of >16 mg/liter), and 1×10^7 (V52-35, TR_{34}/L98H mutation; POS MIC of 0.5 mg/liter) conidia, respectively. The LD_{90} was separately determined for each isolate.

Postinfection viability counts of the injected inocula were determined to ensure that the correct inocula had been injected. The animals were housed under standard conditions, with drink and feed supplied *ad libitum*.

The animal studies were conducted in accordance with the recommendations of the European Community (Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purposes adopted on 22 September 2010), and all animal procedures were approved by the Animal Welfare Committee of Radboud University (RU-DEC 2011-174).

Survival in days postinfection was recorded for each mouse in each group and was considered the primary outcome effect measure to assess the therapeutic efficacy of POS prophylaxis (40). The infected mice were examined at least three times daily. These clinical inspections were carried out in order to ensure that there were no cases of dehydration, torticollis, staggering, high weight loss (a decrease of 15% within 48 h or 20% within 24 h), or drop in body temperature to below 33°C. Mice demonstrating these signs of disease were humanely terminated. On day 15 postinfection, all remaining surviving mice were humanely euthanized under isoflurane anesthesia, and blood and internal organs were collected.

PK analysis of POS prophylaxis in mice. The procedure and pharmacokinetic (PK) parameters for POS prophylaxis are described in our previous study (39). Briefly, a total of 96 outbred CD-1 (Charles River, the Netherlands) female mice, 4 to 5 weeks old, weighing 20 to 22 g, were used to establish immunosuppressed pulmonary infection, as described above. Blood and bronchoalveolar lavage (BAL) samples were drawn at eight predefined time points postinfection (0, 0.5, 1, 2, 4, 8, 12, and 24 h; 3 mice per each time point) and stored at −80°C (39). POS concentrations in plasma and BAL fluid were measured by a validated (for human and mouse matrices) ultraperformance liquid chromatography (UPLC) method with fluorescence detection, as described elsewhere (41). Geometric mean concentrations of total POS in plasma were calculated for each time point ($n = 3$ mice). Peak concentrations in plasma (C_{max}) were directly observed from the data. Pharmacokinetic parameters were derived using noncompartmental analysis with Phoenix, version 6.2 (Pharsight, Inc.). The area under the plasma concentration-time curve (AUC) from time zero to 24 h post infusion (AUC_{0-24}) was determined by use of the log-linear trapezoidal rule. The elimination rate constant was determined by linear regression of the terminal points of the log-linear plasma concentration-time curve. The terminal half-life was defined as ln_2 divided by the elimination rate constant. Clearance (CL) was calculated as dose/AUC_{0-24}. Concentrations of total POS in BAL fluid from three mice per time point were determined as described previously (41). Urea in BAL aspirate and plasma was measured utilizing a modified enzymatic assay (QuantiChrom urea assay kit, DIUR-500; BioAssay Systems) (42, 43). The concentration of POS in epithelial lining fluid (ELF) was then determined by using the ratio of the urea concentration in BAL fluid ($urea_{BAL}$) to that in plasma ($urea_{plasma}$) as described previously (38, 39, 42–47): $drug\ concentration_{ELF} = drug\ concentration_{BAL} \times urea_{plasma}/urea_{BAL}$.

Statistical analysis. All data analyses were performed using GraphPad Prism, version 5.0, software for Windows (GraphPad Software, San Diego, CA). The significance of the differences between growth characteristics of four isolates, including lag phase, first transition phase, log phase, and second transition phase, was determined using one-way analysis of variance (ANOVA), followed by Bonferroni's multiple-comparison test. Mortality data were analyzed by a log rank test. Dose/MIC and AUC_{0-24}/MIC ratios were calculated by dividing the dose (in milligrams per kilogram of body weight) or the AUC by the MIC. The dose, C_{max}, and AUC_{0-24}/MIC ratio data were log_{10} transformed to approximate a normal distribution prior to statistical analysis. The relationship between the *in vivo* prophylaxis (survival), dose, and AUC_{0-24}/MIC was determined by nonlinear regression analysis and the Hill equation, with a variable slope fitted to the data both for each individual isolate and for pooled survival data, with the maximum effect (maximum survival) constrained at ≤100%. The fits were performed for survival data of each strain and all strains simultaneously. The goodness of fit was checked by use of the R^2 value and visual inspection. Statistical significance was defined as a P value of <0.05 (two-tailed). The probability of expected pharmacodynamic (PD) target attainment (AUC/MIC) of POS prophylaxis versus treatment was determined for a range of *A. fumigatus* MICs, as described previously (48). In addition, the 50, 80, and 90% effective pharmacodynamic indices (EI_{50}, EI_{80} and EI_{90}, respectively) of the 24-h area under the concentration-time curve (AUC) of POS best correlating with efficacy were determined. For comparison, an F test was performed to define whether the best-fit values (log 50% effective dose [ED_{50}]) differed between the four groups.

The treatment data used in Fig. 4 was obtained from our previously published study (24), for which an EI_{50} of 184.2 (95% confidence interval [CI], 33.21 to 1,022) was shown to be the optimal pharmacodynamic index to treat disseminated infection caused by the *A. fumigatus* isolate (V52-35) for which the POS MIC was 0.5 mg/liter.

TABLE 2 Pharmacokinetic parameters of POS in plasma and ELF following 3 days of once-daily oral administration of 4, 8, 16, and 32 mg/kg in immunosuppressed mice[a]

POS dose (mg/kg)	C_{max} (mg/liter)		AUC_{0-24} (mg · h/liter)		C_{max} in ELF/C_{max} in plasma ratio (%)	AUC_{0-24} in ELF/AUC_{0-24} in plasma ratio (%)
	Plasma	ELF	Plasma	ELF		
4	5.51	1.58	72.69	14.69	28.68	20.21
8	8.7	2.83	149.80	42.14	32.53	28.13
16	10.92	3.62	198.90	62.23	33.15	31.29
32	15.04	5.32	290.50	78.78	35.37	27.12

[a] For comparison, in humans the C_{max} and AUC_{0-24} values were 0.58 mg/liter and 15.06 mg · h/liter, respectively, with a dosing regimen of 200 mg three times daily (6).

RESULTS

***In vitro* susceptibility and fitness.** The characteristics and *in vitro* susceptibilities of the four *A. fumigatus* isolates are shown in Table 1. All isolates grew well after 48 h of incubation at 35 to 37°C. The three isolates showed increasing POS MICs although the increase from 0.5 mg/liter to >16 mg/liter was not associated with additional mutations in the *cyp51A* gene. Growth curves did not differ in shape and growth rates between the wild-type and two POS-resistant isogenic *A. fumigatus* isolates. Similar growth characteristics were also observed for the nonisogenic isolate harboring the TR_{34}/L98H resistance mechanism (data not shown).

PK of POS. The PK parameters of POS prophylaxis are shown in Table 2. The penetration of POS in ELF based on total drug was between 20.21 and 31.39%. At the range of 4 to 32 mg/kg dosing regiments, the total AUC_{0-24}/MIC was 145.4 to 581 in plasma and 29.38 to 157.56 in ELF for the isolates with MICs of ≤0.5 mg/liter.

As a comparison, the recommended dose of the oral suspension of POS is 200 mg three times a day for the prophylaxis of invasive fungal infections, which corresponds to an AUC of 115.06 mg · h/liter and a C_{max} of 0.58 mg/liter in plasma (6).

Efficacy of POS prophylaxis. (i) Survival curves. Figure 1 shows the survival curves of POS-treated mice by dose. The survival curves for all control groups receiving 0.9% saline orally showed a mortality of 100%. The survival at day 10 postinfection was significantly better for POS-treated mice than for controls (Fig. 1). A dose-response relationship was observed for each isolate. The maximum effect (100% survival) was reached at a dose of 16 mg/kg for the isolates with MICs of ≤0.5 mg/liter, independent of the corresponding genotype. Yet for the isolate with the POS MIC of >16 mg/liter, maximum effect was lower (less than 70%) than for other isolates, even with the highest dose (32 mg/kg).

(ii) Dose-response analysis. The dose-response curves for dosing regimens and control groups of POS prophylaxis are shown in Fig. 2. POS prophylaxis improved the survival of the mice in a dose-dependent manner. A dose-response relationship was observed that depended on the POS dose level but was inde-

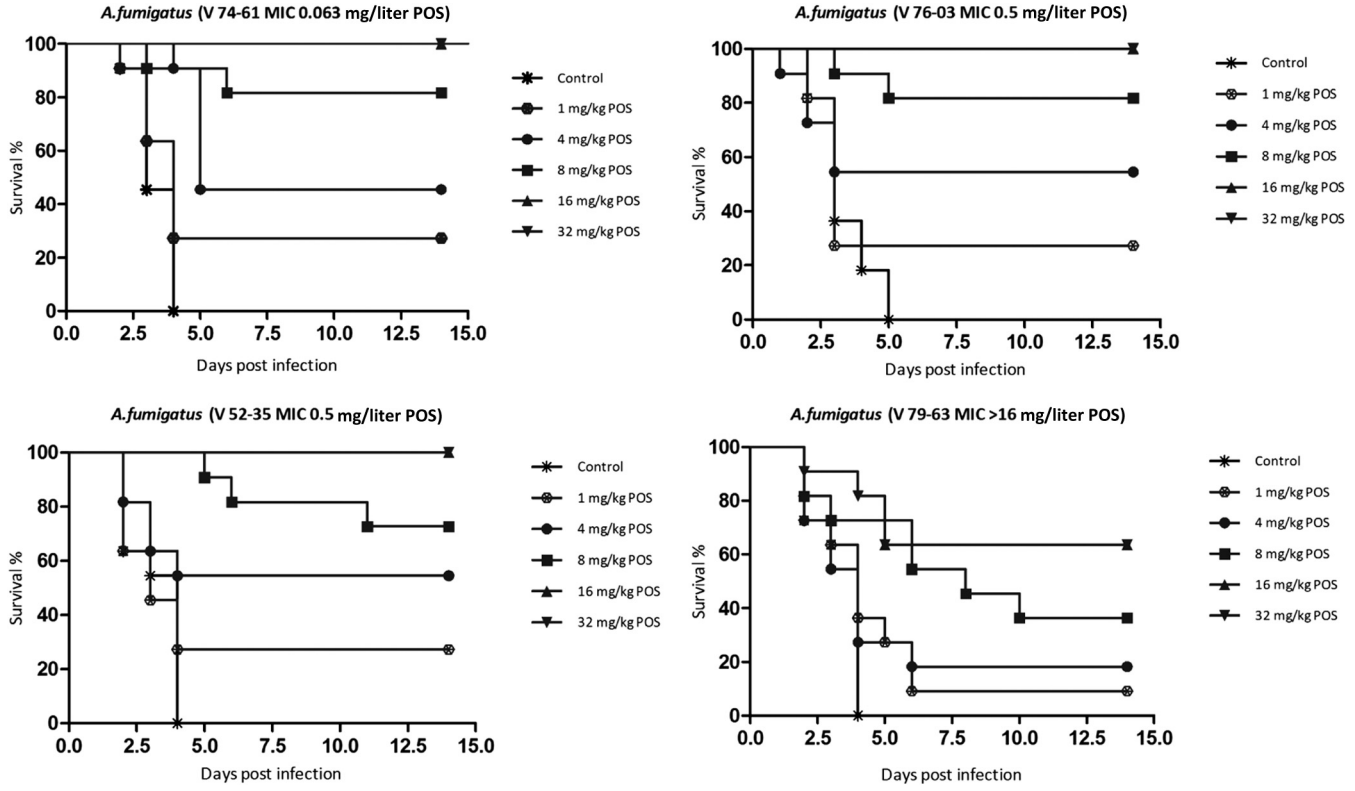

FIG 1 Efficacy of posaconazole prophylaxis against 4 *A. fumigatus* isolates. Three isogenic isolates harboring A9T and F219I point mutations in the *cyp51A* gene with POS MICs of 0.063 (V74-61), 0.5 (V76-03), and >16 mg/liter (V79-63) were obtained sequentially from a patient with aspergilloma under prolonged antifungal therapy. Isolate number 4 (V52-35) was obtained from a patient with proven IA without prior azole exposure and was genetically different, with a POS MIC of 0.5 mg/liter. This isolate harbored a TR_{34}/L98H resistance mechanism in the Cyp51A gene. Animals were treated daily from day −2 to 7 days postchallenge with doses of posaconazole ranging from 1 to 32 mg/kg once daily and observed for 14 days. Placebo groups received only 0.9% saline. For all groups, $n = 11$.

	V 74-61(MIC 0.063 POS)	V 76-03 (MIC 0.5 POS)	V 52-35(MIC 0.5 POS)	V 79-63 (MIC >16 POS)
R^2	0.9565	0.9771	0.9712	0.9552

	V 74-61(MIC 0.063 POS)	V 76-03 (MIC 0.5 POS)	V 52-35(MIC 0.5 POS)	V 79-63 (MIC >16 POS)
ED_{50}	3.350	2.829	3.031	14.08

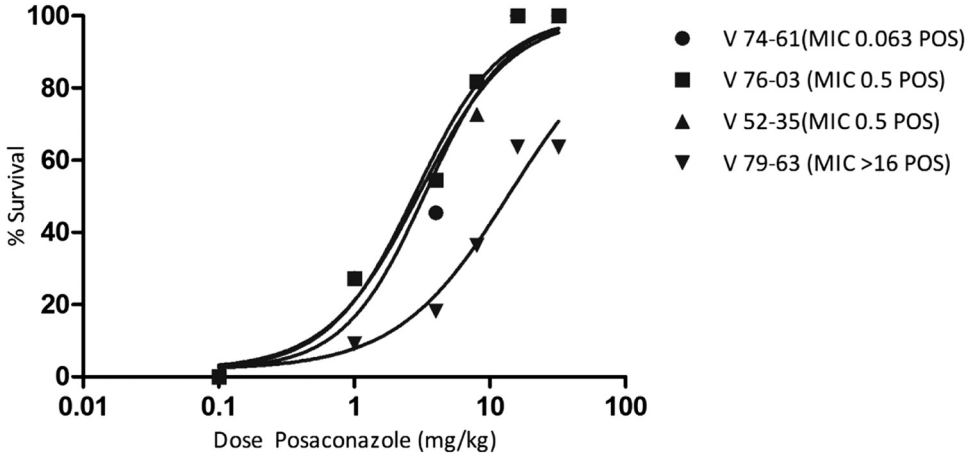

FIG 2 Fourteen-day survival as a function of posaconazole dose against four *A. fumigatus* isolates. Shown are data for the three isogenic isolates harboring A9T and F219I point mutations in the *cyp51A* gene with POS MICs of 0.063 mg/liter (V 74-61), 0.5 mg/liter (V 76-03), and >16 mg/liter (V 79-63) and for a genetically different isolate with a POS MIC of 0.5 mg/liter (V52-35) harboring the TR_{34}/L98H resistance mechanism. POS prophylaxis improved the survival of the infected mice in a dose-dependent manner for all four isolates. The curves indicate fits with the Hill equation for each isolate. ED_{50}, 50% effective dose.

pendent of the genotypes and azole resistance mechanisms. The Hill equation with a variable slope fitted the relationship between the dose and 10-day survival data well, with R^2 values of 0.96 (V74-61, wild type; MIC of 0.63 mg/liter POS), 0.98 (V76-03, F219I mutation; MIC of 0.5 mg/liter POS), 0.95 (V79-63, F219I mutation; MIC of >16 mg/liter POS), and 0.97 (V52-35, TR_{34}/L98H mutation; MIC of 0.5 mg/liter POS), respectively. The 50% effective dose (ED_{50}) was 3.35 mg/kg (95% confidence interval [CI], 2.19 to 5.12 mg/kg), 2.83 mg/kg (95% CI, 1.81 to 4.43 mg/kg), and 14.08 mg/kg (95% CI, 9.06 to 21.90 mg/kg) for three genetically identical isolates with wild type phenotype and low and high POS resistance, respectively, and 3.03 mg/kg (95% CI, 1.91 to 4.80 mg/kg) for the isolate with different genotype and low resistance to POS.

(iii) Exposure-response analysis. The AUC for each dose, determined from PK experiments (Table 2), was used to calculate the AUC_{0-24}/MIC ratio for each isolate, as shown in Fig. 3. The exposure-response relationship had a sigmoidal shape. Increased POS exposure was required to obtain maximum efficacy in mice infected with the isolate with a MIC of >16 mg/liter compared to those infected with the isolate with a MIC of ≤0.5 mg/liter.

The Hill-type model with a variable slope fitted the relationship between the 24-h AUC/MIC ratio and 14-day survival well, with an R^2 value of 0.77 ($P < 0.05$). The 50% effective AUC for POS prophylaxis was 37.38 (95% confidence interval [CI], 7.130 to 196). We also determined the relationship between the *in vivo* efficacy and the peak level C_{max}/MIC (50% effective concentration [EC_{50}] of 1.74; CI, 0.073 to 41.28; R^2 value of 0.76). However, the

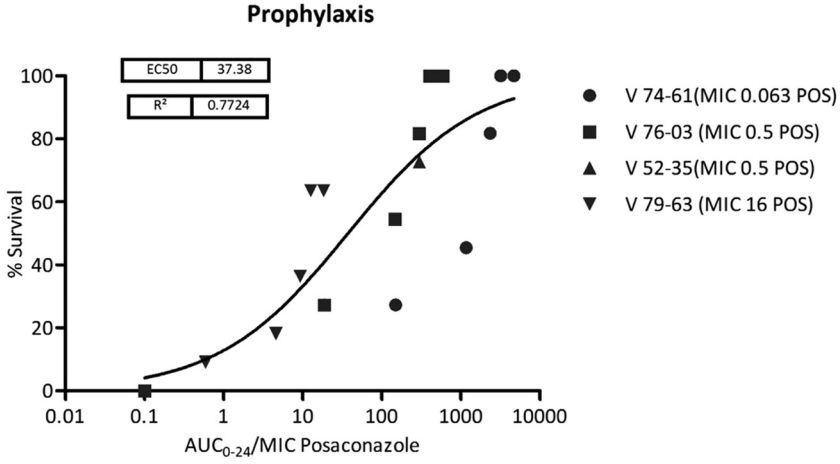

FIG 3 Percent survival as a function of the POS AUC_{0-24}/MIC. The curve is the model fit with the Hill equation for each datum.

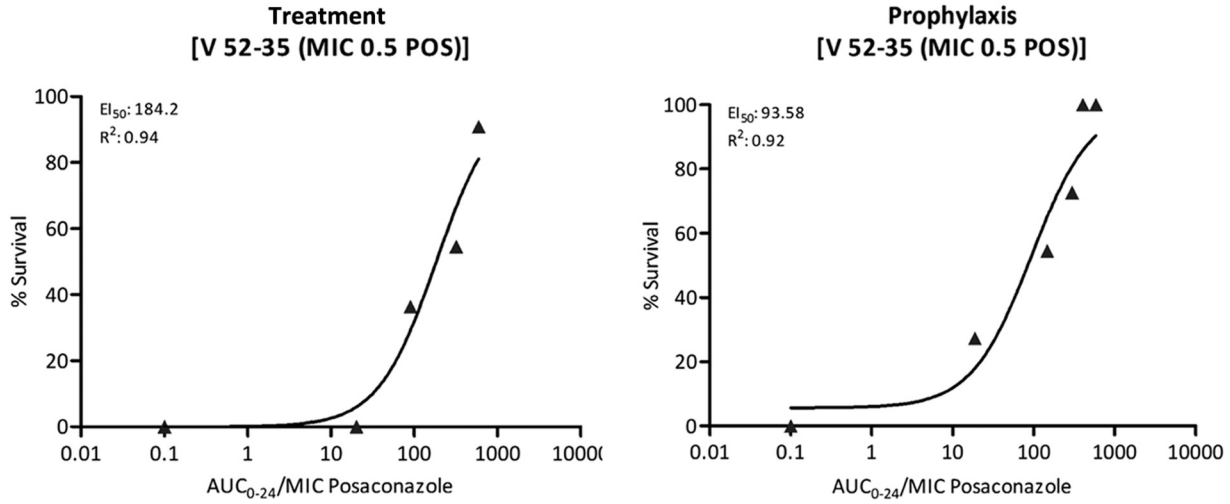

FIG 4 Fourteen-day survival as a function of the posaconazole AUC/MIC ratio for the *A. fumigatus* isolates with a POS MIC 0.5 mg/liter in treatment versus prophylaxis studies. In the current prophylaxis study (right graph), the 50% effective total AUC_{0-24}/MIC was 93.58 (95% CI, 13.90 to 629.9) in order to prevent invasive pulmonary infection caused by the *A. fumigatus* isolate (V 52-35) with the POS MIC of 0.5 mg/liter. The treatment data (left graph) was obtained from our previously published study (24), for which the EI_{50} of 184.2 (95% CI, 33.21 to 1022) was shown to be the optimal pharmacodynamic index to treat disseminated infection caused by the *A. fumigatus* isolate (V 52-35) with the POS MIC of 0.5 mg/liter.

AUC_{0-24}/MIC appeared to be the most important pharmacodynamic index correlating with prophylaxis, which was significantly different between *A. fumigatus* isolates with POS MICs of ≤0.5 mg/liter and the isolate with a POS MIC of >16 mg/liter ($P < 0.05$).

Notably, in the current prophylaxis study, the 50% effective total AUC_{0-24}/MIC was 93.58 (95% CI, 13.90 to 629.9) in order to prevent invasive pulmonary infection caused by the *A. fumigatus* isolate (V 52-35) for which the POS MIC was 0.5 mg/liter. However, in preclinical treatment studies, we have previously shown that a two-times-higher exposure was required to treat infection caused by this isolate (EI_{50} of 184.2 [95% CI, 33.21 to 1,022]) (Fig. 4).

(iv) Comparative efficacy of POS prophylaxis against the four isolates. In order to compare the efficacy of POS prophylaxis in preventing infection caused by the different isolates, the best-fit values for the curves were defined based on the EI_{50}, EI_{80}, and EI_{90} of the AUC of POS and compared to each other (Table 3). The efficacy of POS prophylaxis was significantly different between *A. fumigatus* isolates with POS MICs of ≤0.5 mg/liter and the isolate with a POS MIC of >16 mg/liter ($P < 0.05$). The null hypothesis was rejected in an *F* test ($P = 0.0014$, $F = 10.72$, degrees of freedom, numerator [df_n] = 3; degrees of freedom, denominator [df_d] = 11), indicating that EI_{50} was significantly different (Table 3).

DISCUSSION

Our model indicated that the efficacy of POS prophylaxis in low-resistant isolates (MIC of 0.5 mg/liter) was similar to that of mice infected with the isolate with the wild-type phenotype. In a previous study that investigated the efficacy of POS treatment of azole-resistant isolates, we observed that the efficacy of POS was reduced in mice infected with the isolate harboring the TR_{34}/L98H resistance mechanism (MIC of 0.5 mg/liter) compared to that of mice infected with a wild-type isolate, indicating that higher doses of POS were required to achieve efficacy similar to that for the wild-type-infected mice (24). Our current model indicates that when

TABLE 3 The comparison of efficacy of POS prophylaxis between four *A. fumigatus* isolates based on the EI_{50}, EI_{80}, and EI_{90} values of the 24-h AUC[a]

MIC group and *A. fumigatus* strain (mutation [POS MIC])[b]	EI_{50} (95% CI)	EI_{80} (95% CI)	EI_{90} (95% CI)
MIC ≤ 0.5 mg/liter			
V 74-61 (A9T [0.63])	16.36 (12.18 to 21.97)	31.85 (21.16 to 47.94)	47.03 (26.14 to 84.61)
V 76-03 (A9T F219I [0.5])	13.60 (9.29 to 19.91)	29.18 (18.87 to 45.12)	45.60 (23.40 to 88.88)
V 52-35 (TR_{34}/L98H [0.5])	13.81 (8.92 to 21.38)	33.80 (21.68 to 52.70)	57.06 (28.95 to 112.5)
MIC > 16			
V 79-63 (A9T F219I [>16])	51.22 (38.44 to 68.24)	135.8 (74.38 to 247.9)	240.2 (96.39 to 598.5)
P value[c]	0.0014	0.0002	0.0044
F test (df_n, df_d)	10.72 (3, 11)	17.84 (3, 11)	7.859 (3, 11)

[a] See reference 33.
[b] All MIC values are in mg/liter.
[c] For all isolates, differences between prophylactic efficacy of isolates with MICs of ≤0.5 mg/liter and the isolate with a MIC of >16 mg/liter were significant.

POS is given as prophylaxis, the efficacy against isolates with a POS MIC of 0.5 mg/liter is similar to that of the wild-type isolate.

It has been reported that the POS levels in the lung, at the site of infection/colonization, are relatively high (49, 50), which is consistent with the drug's lipophilic characteristics and its increased intracellular permeability (51, 52). In our model we previously reported high POS levels in epithelial lining fluid, which might explain the high efficacy against low-POS-resistant isolates (38, 39). However, reduced efficacy was observed in isolates with high POS resistance (MIC of >16 mg/liter).

It is unlikely that differences in POS efficacies were due to differences in *A. fumigatus* fitness levels. Although the acquisition of resistance mechanisms during azole therapy has been associated with a fitness cost (53), isolates with resistance mechanisms in the *cyp51A* gene were shown to be as virulent as wild-type controls (54). In our current study we selected three isogenic isolates with increasing POS MICs and demonstrated similar *in vitro* growth characteristics, which indicates that the acquisition of a resistance mechanism was not associated with a fitness cost.

The exposure-response relationships of POS have been defined previously in experimental models of aspergillus infections (24, 25, 55), for which a total AUC_{0-24}/MIC ratio between 167 and 178 was predictive of half-maximal efficacy, given that only the unbound fraction of a drug in serum/plasma is pharmacologically active. Considering the high degree of POS protein binding in plasma (98 to 99%) and negligible protein binding in ELF, the results of the current study indicated that effective local concentrations (less than 50% survival) might be achieved even at the lowest dose (4 mg/kg), with a free AUC_{0-24}/MIC ratio of 1.45 in plasma and 14.69 in ELF. Our model indicates that this level is high enough to prevent infection with *A. fumigatus* isolates with MICs of ≤0.5 mg/liter (Fig. 4). However, for the isolates with higher POS MICs (≥16 mg/liter), the obtained free AUC_{0-24}/MIC was ≤0.18 in plasma and ≤4.92 in ELF at highest dose (32 mg/liter), which indicates the possibility of breakthrough IA. Given that a significant proportion of isolates harboring an azole resistance mechanism exhibit a POS MIC of <0.5 mg/liter (56), the effective exposure is highly probable to be achieved with the recommended dose of POS.

In a recent large international surveillance study, including 22 centers from 19 countries, the proportion of azole-resistant clinical *A. fumigatus* isolates was 3.4% (range, 0 to 26.1% per center) (J. W. M. van der Linden, M. C. Arendrup, A. Warris, K. Lagrou, H. Pelloux, P. M. Hauser, E. Chryssanthou, E. Mellado, S. E. Kidd, A. M. Tortorano, E. Dannaoui, P. Gaustad, J. W. Baddley, A. Uekötter, C. Lass-Flörl, N. Klimko, C. B. Moore, D. W. Denning, A. C. Pasqualotto, C. Kibbler, S. Arikan-Akdagli, D. Andes, J. Meletiadis, L. Naumiuk, M. Nucci, W. J. G. Melchers, and P. E. Verweij, submitted for publication). Among the isolates with an azole-resistant phenotype, 50% exhibited a POS MIC of ≤0.5 mg/liter which according to our model could be prevented with POS prophylaxis. Given the above-mentioned epidemiology, in centers that choose to give POS prophylaxis to high-risk patients, the probability of breakthrough infection due to azole-resistant *A. fumigatus* appears to be very low.

Notably, the size of inoculum used for infection in the current preclinical study may not seem to represent human infections. However, the concordance of PK/PD index magnitudes obtained from this model and humans has been demonstrated in terms of efficacy and pharmacokinetics of antifungals against *A. fumigatus* infections, using survival as primary endpoint (22–24, 34, 39, 57). Thus, the results of our study can be useful in a human setting.

We conclude that POS is effective in preventing invasive pulmonary aspergillosis in our immunocompromised mouse model. However, our model indicates that treatment does not prevent the full range of POS resistance phenotypes. Possibly higher exposure can be obtained using the new formulations, i.e., a POS tablet, which may then also enable prevention of infection due to *A. fumigatus* isolates highly resistant to POS.

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